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# NUCLEAR MAGNETIC RESONANCE IDENTIFICATION OF MILITARY NERVE AGENTS AND RELATED COMPOUNDS BY TWO-DIMENSIONAL $^{31}P$-$^{1}H$ HETERONUCLEAR OVERHAUSER EFFECT SPECTROSCOPY

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Triethylphosphate and two diastereoisomers of *O*-pinacolyl methylphosphonic acid, the primary hydrolysis produet of the nerve agent soman, were identified in an organie solvent with a single $^{31}$P-$^{1}$H heteronuclear Overhauser effect spectroscopy (HOESY) spectrum correlating phosphorus nuclei to their *J*-coupled protons. An analogous heteronuclear multiple bond correlation experiment is also presented to demonstrate the superiority of HOESY for detecting long-range $^{31}$P-$^{1}$H pairs. This superiority results from two features specific to the HOESY technique, its reliance on the transfer of phase coherence through conformational space, and its direct detection of $^{31}$P nuclei.

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## 1. INTRODUCTION

As demonstrated by the 1995 poison gas attack in a Tokyo subway station, chemical weapons pose a threat not only to U. S. and allied militaries but innocent civilians as well. The weapons are attractive to foreign states or terrorists seeking a mass-destruction capability because they are relatively inexpensive to produce and do not require the elaborate infrastructure needed for nuclear weapons. As with any small molecule analyte, chemical weapons are typically identified in environmental, forensic or intelligence samples by conventional analytical techniques relying on spectral libraries or chromatographic retention times. These methods, however, do not always lead to a positive identification of a chemical weapon or related compound. Moreover, impurities that can be valuable in determining which government, country, or terrorist group has produced the weapon usually do not appear in spectral libraries. In such cases, the elucidation of analyte molecular structure by interpretative means may present the best means for positive identification. Although several techniques can provide valuable information for determining molecular structure, nuclear magnetic resonance (NMR) spectroscopy is probably the single most powerful technique for providing detailed and unambiguous structural information within a reasonable timeframe. Although more expensive and less sensitive than conventional techniques, NMR spectroscopy is less involved because it does not require extensive sample preparation and generates results that are easily interpreted. Over the years, $^{1}H$- and $^{13}C$-NMR spectroscopy has emerged as important analytical tools for chemical weapons and their related compounds.

Particularly for nerve agents and other organophosphorus compounds, $^{31}P$ NMR spectroscopy is often is used to supplement $^{1}H$- and $^{13}C$-NMR data. In addition to the wide range of $^{31}P$ chemical shift values, the 100% natural abundance of the $^{31}P$ nucleus and its high sensitivity, which is only ~15 times less sensitive than that of a $^{1}H$ nucleus, make $^{31}P$ NMR experiments a reliable approach for investigating phosphorus-containing analytes $\geq 1$ μmolar. These properties are in sharp contrast to the low natural abundance and sensitivity of the $^{13}C$ nucleus, which is also characterized by long relaxation times, and the severe signal overlap often encountered in $^{1}H$ spectra. Several two-dimensional techniques have been developed to observe $^{1}H$ nuclei coupled to a $^{31}P$ nucleus, while effectively suppressing unwanted background signals at the same time. These typically give spectra with very good information content, and in some cases, unambiguous identification of analytes are possible. For scalar coupled $^{1}H$-$^{31}P$ pairs ($J_{HP}$), the heteronuclear multiple quantum correlation (HMQC) technique[1-3] and the heteronuclear multiple bond correlation (HMBC) technique[4] are available and in wide use today. Both are $^{1}H$-detected, zero- and double-quantum correlation techniques, however, only the HMBC pulse sequence is optimized for long-range scalar coupling. Correlations in HMQC and HMBC spectra generally represent $^{1}H$ and $^{31}P$ nuclei separated by 1-3 covalent bonds. Two-dimensional techniques for observing dipolar coupled $^{1}H$-$^{31}P$ pairs, on the other hand, are limited to the heteronuclear nuclear Overhauser effect spectroscopy (HOESY) technique.[5,6] Correlations in

these spectra arise from cross-relaxation between dipolar-coupled $^{1}H$-$^{31}P$ nuclei, and their correlations result from phase coherence transfer through conformational space between the nuclei, with their intensities directly proportional to the distance separating them. When considering literature articles cited over the past 15 years, HOESY does not appear to be used as extensively as the HMQC and HMBC techniques for correlating $^{1}H$ and $^{31}P$ nuclei. Despite this observation, we have found HOESY spectra to be superior to HMQC and HMBC spectra for $^{1}H$-$^{31}P$ correlations in organophosphorus compounds. Herein, we illustrate this finding with an environmental sample suspected to contain chemical nerve agents by comparing data from HMBC and HOESY experiments.

## 2. EXPERIMENTAL PROCEDURES

### 2.1 Chemicals, Solvents, and Supplies.

$CDCl_3$ (≥99% isotope enrichment), tetramethylsilane, and phosphoric acid were purchased from Aldrich (Milwaukee, WI). NMR sample tubes were purchased from Wilmad-Labglass (Buena, NJ).

### 2.2 Sample Preparation.

A 1 mL aliquot of an environmental sample containing an organic solvent was prepared for NMR analysis by addition of 100 µL $CDCl_3$ for use as a lock solvent. The resulting solution was placed into a 5-mm NMR sample tube just prior to NMR analysis. (**Warning:** Chemical weapons can cause chronic health problems such as cancer, severe bodily injury, or death to laboratory personnel, and must be handled carefully. Samples containing all but trace amounts of chemical weapons must be handled in a closed system or a fume hood with a minimum airflow velocity of 100 ft/min).

### 2.3 NMR Spectroscopy.

All NMR spectroscopy was conducted at 11.75 Tesla, using an Avance DRX-500 spectrometer (Bruker-Biospin Corporation, Billerica, MA) with XWIN-NMR software (version 3.1) for data acquisition and processing. The spectrometer was fitted with a triple resonance TXI probehead (Bruker-Biospin Corporation) containing dedicated $^{1}H$, $^{13}C$, and $^{31}P$ channels (inverse configuration). Experiments were conducted at 25.0 ± 0.2 °C, with the sample spinning at 20 Hz for of one-dimensional experiments only. For two-dimensional spectroscopy, quadrature was used exclusively for collecting complex data in the direct dimension ($t_2$), while different phase cycling schemes used for the indirect dimension ($t_1$) gave either real or complex data. All $^{1}H$ and $^{31}P$ decoupling used the WALTZ-16 composite pulse sequence.[7]

#### 2.3.1 Routine NMR Spectroscopy.

$^{1}H$ free induction decay data of 16,384 or 65,536 complex points were recorded from the summation of 32 acquisitions using 10-12 ppm spectral windows, 90° pulse widths, and 5-9 s relaxation delays. These data were Fourier transformed directly into spectra and manually

adjusted into pure absorption mode. $^{1}H$ chemical shifts were referenced to external tetramethylsilane.

For $^{1}H$-decoupled $^{31}P$ spectra, free induction decay data of 65,536 complex points were recorded from the summation of 128 acquisitions using 220 ppm spectral windows, 90° pulse widths and 20 s relaxation delays. Recorded data were multiplied by an exponential window function (apodized) with a line-broadening factor of 2 Hz before Fourier transformation into spectra and manual adjustment into pure absorption mode. $^{31}P$ chemical shifts were referenced to external 85% phosphoric acid.

### 2.3.2 $^{1}H$-$^{31}P$ HMBC Spectroscopy.

$^{31}P$ nuclei were correlated to their *J*-coupled $^{1}H$ nuclei using a pulse sequence incorporating the pulsed-field gradient selection of heteronuclear zero and double quantum coherence and a delay for the evolution of long-range $J_{PH}$-couplings.[8] The data set was collected as a 1024 real x 8192 complex matrix with 16 $^{1}H$ acquisitions per $t_1$ increment, each using 2 s relaxation delays, 4.7 ppm spectral windows in the $^{1}H$ dimension ($t_1$), and a 39 ppm window in the $^{31}P$ dimension ($t_2$). $^{1}H$ data were multiplied by a trapezoidal window function prior to Fourier transformation. For the indirect $^{31}P$ dimension, data were expanded to 2048 real points with linear prediction,[9] and multiplied by a sine-squared window function before final Fourier transformation into a 2048 x 4096 real, magnitude mode spectrum.

### 2.3.3 $^{31}P$-$^{1}H$ HOESY.

$^{31}P$ nuclei were correlated to dipolar-coupled $^{1}H$ nuclei using a HOESY pulse sequence[6] incorporating TPPI to achieve quadrature in the indirect dimension. The data set was a 512 x 8192 complex matrix with a 4.7 ppm spectral window in the $^{1}H$ dimension ($t_1$), and a 39 ppm window in the $^{31}P$ dimension ($t_2$). Each $t_1$ increment derived from eight $^{1}H$-decoupled $^{31}P$ acquisitions using 1.5 s mixing times and 16 s relaxation delays. These were apodized with a line-broadening factor of 5 Hz before Fourier transformation. $^{1}H$ data were extended to 2048 real points by linear prediction,[9] and then multiplied by a sine window function before Fourier transformation into a phase-sensitive spectrum of 2048 x 4096 real points.

## 3. RESULTS AND DISCUSSION

An unidentified sample suspected to contain chemical nerve agents was characterized by several analytical techniques, including NMR spectroscopy. $^{19}F$ analysis of the sample failed to detect any signals, while a $^{31}P$ experiment identified signals at 0.10 and 32.31 ppm, as well as a much smaller signal at 32.68 ppm (data not shown). The results demonstrate the presence of 1-3 phosphorus compounds in the sample, with none containing a fluorine atom. Further analysis by $^{1}H$ spectroscopy produced the spectrum shown in Figure 1 with ≥10 signals at various intensities. Close examination of the spectrum clearly reveals two sets of doublet signals at ~1.24 ppm with *J*-coupling constants of 18.6 Hz each, indicative of methylphosphonate groups [$CH_3$-P(O)(OR)(OR'), R and R' may or may not be identical]

common to many military nerve agents. As shown in Figure 2, the more intense of these signals was correlated to the 32.31ppm $^{31}P$ signal by $^{1}H$-$^{31}P$ HMBC spectroscopy. The spectrum correlates three other $^{1}H$ signals to this $^{31}P$ signal; a 0.67 ppm signal appearing as a singlet (d in Figure 2), most likely from ≥1 methyl groups, a 1.03 ppm signal appearing as a doublet (b in Figure 2), and a multiplet signal at 3.95 ppm (c in Figure 2). The chemical shift of the latter signal and its octet (a doublet of quartets) nature implicates it as representing a methylene or methine group bonded to an oxygen atom of the methylphosphonate group, as well as a single methyl group. Considered with the 0.67 ppm signal appearing to be isolated from $J_{HH}$-coupling, the three $^{1}H$ signals can be rationalized with a single pinacolyl group [-OCH($CH_3$)(C($CH_3$)$_3$)]. This tentatively identifies the analyte as *O*-pinacolyl methylphosphonic acid [$CH_3$-P(O)(OH)(*O*-pinacolyl)], which is the primary hydrolysis product of the nerve agent soman. In contrast, the HMBC spectrum correlates the 0.10 ppm $^{31}P$ signal to only two $^{1}H$ signals: a 1.09 ppm triplet signal most likely representing a methyl group bound to a methylene group and a 3.88 ppm multiplet. The chemical shifts of the multiplet signal and the 0.10 ppm $^{31}P$ signal suggest that both $^{1}H$ signals represent an *O*-ethylphosphate analyte [(*O*-ethyl)P(O)(OH)$_2$, (*O*-ethyl)$_2$P(O)(OH), or (*O*-ethyl)$_3$P(O)]. Matrix spike additions to other aliquots of the sample verified the analytes as *O*-pinacolyl methylphosphonic acid and triethylphosphate [(*O*-ethyl)$_3$P(O)], respectively. The HMBC spectrum failed to correlate the 32.31 ppm $^{31}P$ signal to even one $^{1}H$ signal. An $^{31}P$-$^{1}H$ HOESY experiment was conducted. The resulting spectrum is presented in Figure 3 with its correlations displayed at similar intensities to those in Figure 2. Casual examination of the spectra reveals that the HOESY correlations are much more uniform in intensity than those of the HMBC correlations. As a consequence, long-range correlations (those from nuclei separated by >3 covalent bonds) are significantly larger in the HOESY spectrum. This is best exemplified by the two correlations for the methyl signals of *O*-pinacolyl methylphosphonic acid (b and d in Figures 2 and 3). Expansion of the HOESY spectrum as shown in Figure 4 reveals correlations between the 32.68 ppm $^{31}P$ signal and four $^{1}H$ signals, one of which is the less intense doublet signal at ~1.24 ppm already implicated as a methylphosphonate group (a in Figure 4). The remaining three correlations represent $^{1}H$ signals that are completely obscured by *O*-pinacolyl methylphosphonic acid and triethylphosphate signals in Figure 1; however, their HOESY correlations illustrate that each is similar in chemical shift to one of the three $^{1}H$ signals of the *O*-pinacolyl group from the pinacolyl methylphosphonic acid. It is reasonable to assume that the 32.31 ppm $^{31}P$ signal represents the minor diastereoisomer of *O*-pinacolyl methylphosphonic acid, which was validated by matrix spike addition. All remaining $^{1}H$ signals such as those at 1.39, 2.33, 2.46, and 3.29 ppm were found to derive from compounds, other than chemical weapons, that do not contain phosphorus atoms.

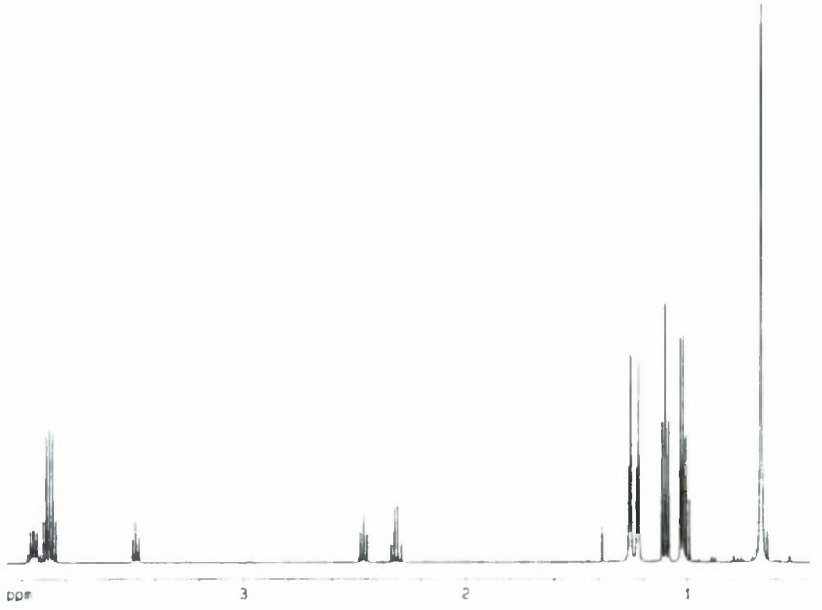


Figure 1. $^{1}H$ Spectrum of the Organic Sample.

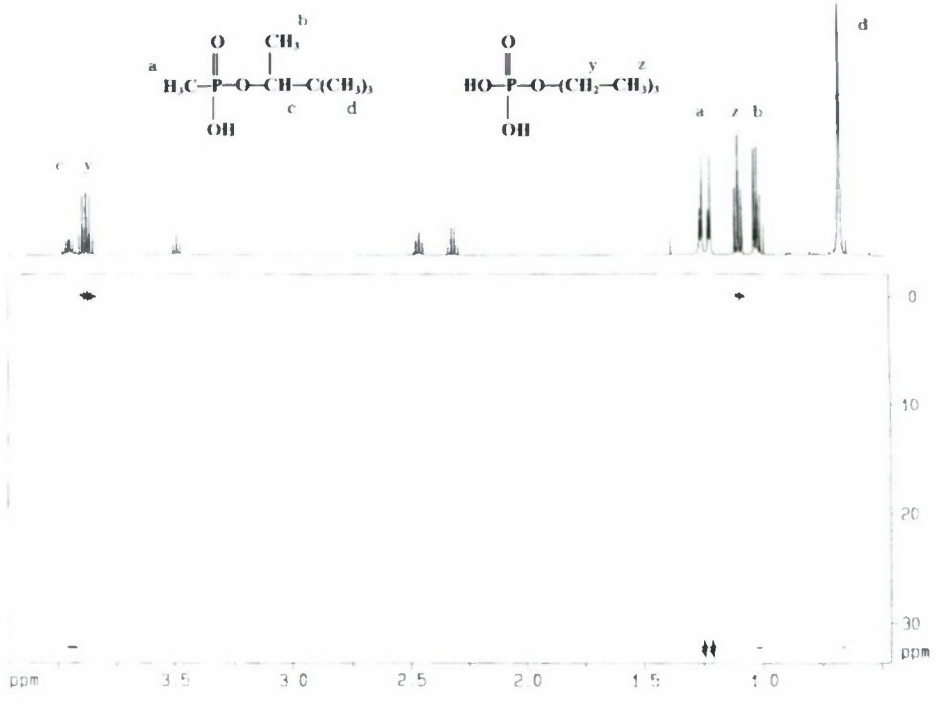


Figure 2. $^{1}H$-$^{31}P$ HMBC Spectrum of the Analytical Sample and Corresponding $^{1}H$ Spectrum. $^{1}H$ signals for *O*-pinacolyl methylphosphonic acid (top left) are identified with letters **a** through **d**, whereas those for triethylphosphate (top right) are identified as **y** and **z**.

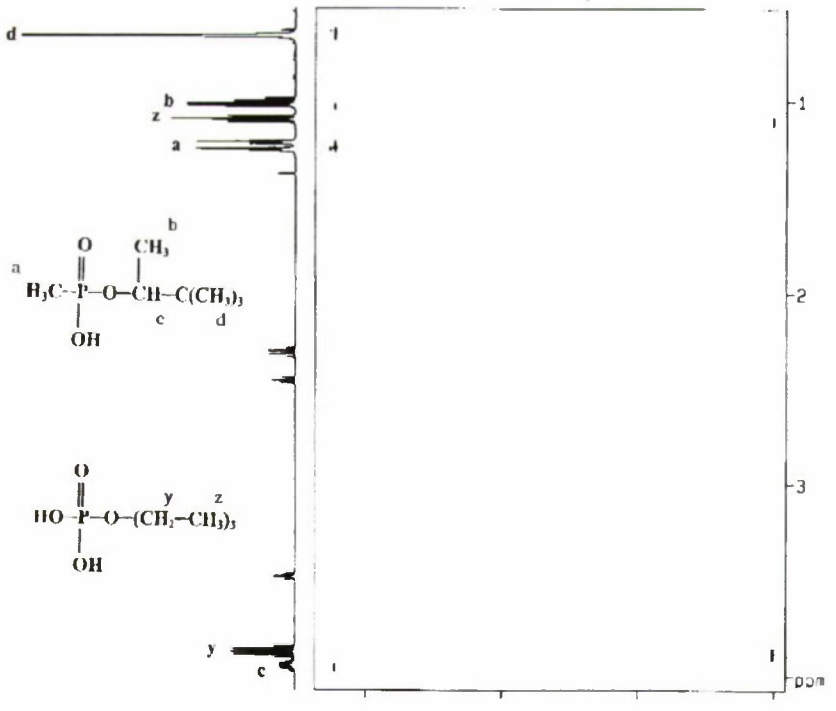


Figure 3. $^{31}P$-$^{1}H$ HOESY Spectrum of the Analytical Sample. The corresponding $^{1}H$ spectrum is included for reference.

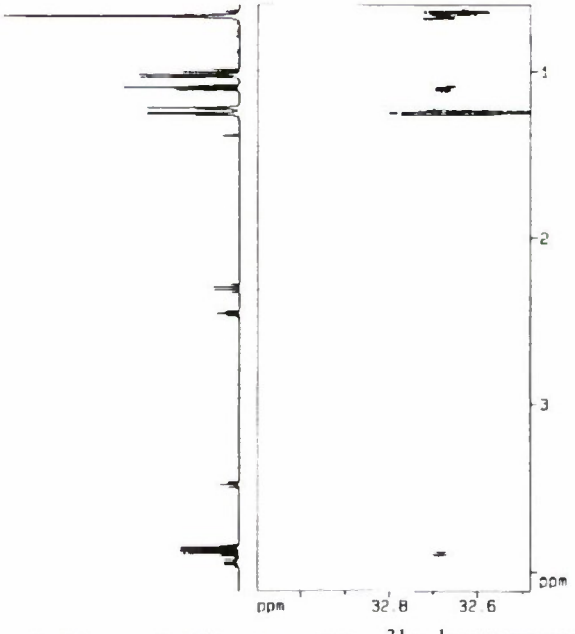


Figure 4. Expanded Region of the $^{31}P$-$^{1}H$ HOESY Spectrum and Corresponding $^{1}H$ Spectrum.

These results illustrate the superiority of the HOESY technique for identifying $^{1}H$-$^{31}P$ correlations in organophosphorus compounds. From our observations, HOESY not only detects more correlations than the HMQC and HMBC technique, but also is better suited for detecting long-range $^{1}H$-$^{31}P$ pairs. This superiority results from two features unique to the HOESY technique, which are its reliance on the transfer of phase coherence through conformational space and its direct detection of $^{31}P$ nuclei.

### 3.1 Transfer of Phase Coherence Through Conformational Space and Conformational Flexibility.

As expected from their small size, chemical nerve agents and other organophosphorus compounds are conformationally flexible in solution at ambient temperatures.[10-12] This flexibility allows many of their $^{1}H$ nuclei to spend at least some time in close proximity to their $^{31}P$ nucleus. Moreover, because HOESY relies on the transfer of phase coherence, through conformational space, between dipolar-coupled heteronuclei, even long-range $^{1}H$-$^{31}P$ pairs can dipolar-couple long enough to generate a correlation. In contrast, the HMQC and HMBC techniques are based on $J_{PH}$-coupling for detecting $^{1}H$-$^{31}P$ pairs, the magnitude of which decreases proportionally as a function of the number of covalent bonds separating the nuclei. The probability of detecting $^{1}H$-$^{31}P$ pairs decreases inversely with $^{1}H$-$^{31}P$ internuclear distance, which is not the case for HOESY. This is clearly illustrated in Figure 5, where a $^{1}H$ spectrum extracted from the HOESY data set (one column from the HOESY spectrum) corresponding to the $^{31}P$ signal for the more populated diastereoisomer of *O*-pinacolyl methylphosphonic acid is compared to the analogous spectrum from the HMBC data set (one row from the HMBC spectrum). The methylphosphonate signals (a in the figure) are the most intense in both spectra simply because their $^{1}H$ nuclei are the closest to the $^{31}P$ nucleus (separated by only two covalent bonds) and are the most likely $^{1}H$-$^{31}P$ correlations to be detected. The largest difference between the two spectra is the intensity of the signal for the nine equivalent methyl $^{1}H$ nuclei (d in the figure) relative to that of the respective methylphosphonate signal in the same spectrum; this is much greater in the HOESY spectrum. These nine methyl $^{1}H$ nuclei and the $^{31}P$ nucleus are separated by more covalent bonds (five total) than any other $^{1}H$ nuclei in the molecule, representing the most difficult to correlate to the $^{31}P$ nuclei by HMBC or any technique relying on $J_{PH}$-coupling. Similar arguments can be presented for the intensities of the methine $^{1}H$ signal (c in Figure 5). These intensity differences relate directly to the superiority of the HOESY technique for measuring long-range $^{1}H$-$^{31}P$ correlations. More correlations are typically observed when using HOESY experiments rather than adding TOCSY spin-locks to HMBC and HMQC pulse sequences, which has the disadvantage of decreasing sensitivity for short-range correlations.

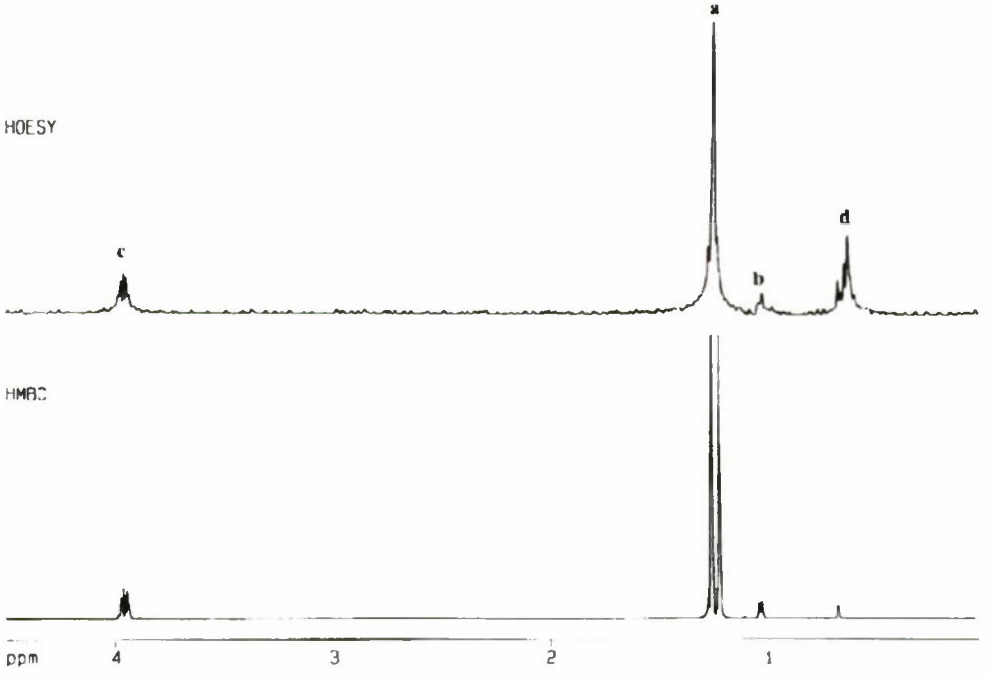


Figure 5. $^{1}$H Spectrum from the $^{31}$P-$^{1}$H HOESY Data Set Corresponding to the 32.31 ppm $^{31}$P Signal and the Analogous Spectrum from the $^{1}$H-$^{31}$P HMBC Data Set.

The HOESY experiment is based on the $^{1}$H-$^{31}$P NOE, which does not suffer from the nulling effect of the homonuclear case. However, the spin-lattice relaxation rate ($T_1^{-1}$) of non-protonated $^{31}$P nuclei often has a significant component due to the chemical shift anisotropy mechanism, the rate of which increases with the square of the magnetic field strength. At high field strengths, therefore, the NOE available for non-protonated $^{31}$P nuclei may be significantly less than the maximum expected from pure dipolar relaxation. From previous experience, this has not been problematic at either 7.06 or 11.75 Tesla, but it has been observed that HOESY experiments appear more sensitive at the lower field strengths. Kövér and Batta[13] have presented formulas for estimating the optimum mixing time for measuring maximum NOE correlations in a two-spin system. For $^{1}$H-$^{31}$P pairs in organophosphorus compounds, this is found to be approximately $2T_{1(1H)}$.

## 3.2 Direct Detection of $^{31}$P Nuclei.

Another contributing factor to the superiority of HOESY for detecting $^{1}$H-$^{31}$P correlations is its $^{31}$P observation in the direct dimension, rather than the $^{1}$H observation in HMBC and HMQC experiments. Signals often overlap in $^{1}$H spectra, which can cause data points in the indirect dimension to be modulated by two (or more) signals rather than a single signal. Particularly when overlapping signals are of very different intensities, the modulation is dominated by the most intense signal, which can significantly compromise modulation of the less intense signal. The broad range of $^{31}$P chemical shifts severely reduces chances for overlapping signals. This problem is rarely encountered with HOESY. The correlations shown in the

HOESY spectrum of Figure 4 could not be detected with the HMBC experiment described above, even with its higher sensitivity from $^{1}H$ detection.

### 3.3 Phase-Sensitive $^{31}P$-$^{1}H$ Spectra.

In addition to its superiority for detecting $^{1}H$-$^{31}P$ correlations, the HOESY technique provides a mechanism to generate phase-sensitive, two-dimensional, $^{1}H$-$^{31}P$ spectra. Unlike $^{1}H$-$^{13}C$ or $^{1}H$-$^{15}N$ correlation spectroscopy, experiments correlating $^{1}H$ and $^{31}P$ nuclei by $J_{PH}$-coupling cannot give phase-sensitive spectra with all correlations in pure absorption mode. The most likely explanation for this is that because $J_{HH}$-coupling constants are in the same range as typical $J_{PH}$-coupling constants (0.2-10 Hz), $J_{HH}$-couplings evolve during the delay periods of the pulse sequences in addition to the desired $J_{PH}$-couplings, introducing phase-related artifacts from $J_{HH}$-coupling into the phase sensitive data sets. These two-dimensional $^{1}H$-$^{31}P$ correlation experiments are usually presented as magnitude mode spectra to eliminate the negative components of the correlations, often resulting in correlations with reduced sensitivity or resolution. Because of this, $^{1}H$-$^{31}P$ correlation experiments are usually based on magnitude mode HMQC or HMBC pulse sequences that do not generate phase-sensitive data.

## 4. CONCLUSIONS

Heteronuclear nuclear Overhauser effect spectroscopy (HOESY) has been demonstrated to be superior to other two-dimensional nuclear magnetic resonance techniques such as heteronuclear multiple quantum correlation and heteronuclear multiple bond correlation, which exploit $J_{PH}$-coupling for correlating $^{31}P$ nuclei to $^{1}H$ nuclei in military nerve agents and other organophosphorus compounds. The HOESY technique has been shown to detect more $^{31}P$-$^{1}H$ correlations and is much better suited for detecting long-range correlations as well. This superiority is attributed to two features specific to the HOESY technique, which are its dependence on the transfer of phase coherence through conformational space for detecting longer-range $^{1}H$-$^{31}P$ pairs and its direct detection of $^{31}P$ nuclei for eliminating artifacts from overlapping signals typically encountered in the other techniques. Finally, HOESY provides the only means to generate phase-sensitive $^{1}H$-$^{31}P$ spectra, resulting in improved detection of $^{1}H$-$^{31}P$ correlations and spectral resolution.

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